cholesterol in vivo. Liquid-crystalline liposomes were more effective in mobilizing cholesterol than gel-state liposomes.

Cholesterol and [4-(2-hydroxyethyl)]-piperazineethanesulfonic acid (Hepes) were obtained from Sigma. [^{14}C]cholesterol hexadecyl ether and [^{3}H]cholesterol were purchased from New England Nuclear. Egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG) and egg phosphatidylglycerol (EPG) were supplied by Avanti Polar Lipids. Bio-Gel A-15m medium was purchased from Bio-Rad. All chemicals, thin layer chromatography plates and solvents were of analytical grade and purchased from BDH Chemicals.

All liposome preparations were labelled using trace amounts of [^{14}C]cholesterol hexadecyl ether (CHE). This labelling is useful as (1) it does not undergo passive exchange between membranes; (2) mice do not exhibit cholesterol-ester exchange protein activity; and (3) the ether-linked fatty acid is not cleaved in the plasma. Consequently, in this model system CHE is an excellent liposome marker and vesicle concentrations in the plasma were estimated from the specific activity of this label.

A chloroform solution of EPC and [^{14}C]CHE was vortexed and solvent was removed under a stream of N_2. The sample was dried under high vacuum for 2 h. The dry lipid film was hydrated in 150 mM NaCl, 20 mM Hepes (pH 7.4) to generate multilamellar vesicles (MLV). Vesicles were prepared from MLV either by sonication, to generate small unilamellar vesicles (SUV) or extrusion to produce large unilamellar vesicles (LUV). Sonication was performed using a Branson tip sonifier, following standard protocols. The MLV suspension was diluted to 30 mg/ml, immersed in an ice bath and subjected to 3 cycles of sonication, each of 10-min duration. The initial milky suspension became clear and the vesicle size was 30 nm, as determined by quasi-elastic light scattering (QELS). The SUV were centrifuged at 10000Xg for 30 min to remove titanium fragments originating from the sonicator tip.

Extrusion was carried out using a 10 ml Lipex Biomembranes Extruder equipped with a water jacketed

thermobarrel as described by Hope et al., Biochim. Biophys. Acta, 812:55-65 (1985), incorporated herein by reference. MLV were sized through two stacked polycarbonate filters of defined pore size to generate a variety of LUV and homogeneous MLV as described in Hope et al., supra, and Mayer et al., Biochim. Biophys. Acta, 858:161-168 (1986), incorporated herein by reference.

The size of vesicles generated by sonication and extrusion procedures was determined by QELS analysis utilizing a Nicomp Model 370 submicron laser particle sizer equipped with a 5-mW He-Ne Laser. The Nicomp QELS analyzes fluctuations in light-scattering intensities due to vesicle diffusion in solution. The measured diffusion coefficient is used to obtain the average hydrodynamic radius and thus, the mean diameter of vesicles. The following diameters are expressed as the mean ±S.D. of vesicle preparations prior to injection. Vesicles prepared by sonication were 30±7 nm in diameter (SUV_{30}). Vesicles prepared by extrusion through filters with a pore size of 0.05 μm were 70±19 nm, 0.1 μm pore size were 125±30, and 0.4 μm pore size were 237±90 nm. Generally, the vesicles prepared by extrusion are referred to herein by the filter pore size used in their preparation, i.e., LUV_{50}, LUV_{100} and MLV_{400}.

Female BDF-1 or CD-1 mice, weighing 20-22 g (Sprague-Dawley), were used throughout this study. Liposomes were injected via the tail vein at a dose of 300 mg/kg, which was typically 6 mg of liposomes in 200 ml of buffer injected for each animal. Control mice were injected with an equal volume of buffer and both groups were sacrificed at specified times with blood collection in EDTA microtainer tubes by heart puncture. Plasma was obtained following centrifugation at 2000 x g for 10 min, and an aliquot removed for scintillation analysis using a Beckman LS 3801 liquid scintillation counter. The average of data from 16 mice (from four separate experiments) is indicated at each time point, unless indicated otherwise.

A 27 x 1.5 cm Bio-Gel A-15m gel filtration column, equilibrated with 150 mM NaCl, 10 mM Tris, 0.1% EDTA, 0.3%

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NaN_3 (pH 7.4) was used to fractionate plasma samples. Columns were eluted at a flow rate of 1 ml/min and 1-ml fractions were collected for radioactivity and lipid analyses. Data on the cholesterol:phospholipid (C/P) ratio of vesicles and lipoproteins after infusion was obtained from pooled fractions corresponding to the liposomal and lipoprotein peaks. The Bio-Gel columns were calibrated with respect to lipoprotein elution by preparing purified human lipoprotein fractions using standard ultracentrifugation procedures as described in Schumaker et al., Methods Enzymol., 128:155-181 (1986), incorporated herein by reference. The lipoprotein fractions were each labelled with [^{3}H]cholesterol. The elution profiles of the columns were monitored for radioactivity.

Pooled column fractions and plasma samples were extracted employing the Bligh and Dyer procedure. Bligh and Dyer, Can. J. Biochem. Physiol., 37:911-917 (1959), incorporated herein by reference. The lipid extracts were analyzed for total cholesterol using the assay method of Rudell and Morris, J. Lipid Res., 14:364-366 (1973). Free and esterified cholesterol concentrations were determined following separation by TLC using hexane/ether/acetic acid (70:30:1 (v/v)). Standards were used to identify the area of the plate corresponding to these two lipids, the silica was aspirated and the lipid eluted for assay using chloroform/methanol (2:1 (v/v)). Plasma vesicle phospholipid content was determined by dividing [^{14}C]CHE radioactivity by liposome-specific activity and phospholipid concentrations were determined by the method of Fiske and SubbaRow, J. Biol. Chem., 66:375-400 (1925). Erythrocytes were extracted using the method of Rose and Oklander (J. Lipid Res., 6:428-431 (1965)), followed by a Bligh and Dyer wash to remove residual salts. An aliquot of red blood cells was retained for cell number determination using a Coulter cell counter in order to express cholesterol and phospholipid concentrations as mmol/10^9 cells.

Blood was pooled from a group of mice and red cells packed by low-speed centrifugation. The serum was labelled with [^{3}H]cholesterol by incubation for 10 min at 37°C with 100

μCi of radioisotope dried from ethanol. The labelled serum was added to the packed cells and the mixture incubated at room temperature for 30 min. The cells were washed and approximately 10^6 dpm of [^{3}H]cholesterol-labelled cells injected into the experimental groups via the tail vein. Approximately 1 min after the injection of cells, saline or liposomes were administered.

Donor and acceptor liposomes were separated employing ion exchange chromatography. A 10-fold excess of donor vesicles (100 nm diameter) composed of EPC/EPG/Chol (40:15:45 molar ratio) were incubated with 100-nm or 400-mm EPC acceptors. Donor liposomes were labelled with [^{3}H]cholesterol at 5 μCi/100 mg total lipid and acceptors were labelled with [^{14}C]CHE at 0.5 μCi/100 mg lipid. At specified time intervals, 50 μl aliquots of the incubation mixture (1 mg acceptor + 10 mg donor/ml) were removed and passed down a DEAE-Sepharose 6B-CL column prepared in a 1-ml tuberculin syringe equilibrated with 30 mM NaCl, 20 mM Hepes (pH 8.0). Columns were spun at 1000 x g for 1 min prior to applying aliquots of the incubation mixture. The liposome mixture was spun through the column and the eluant (acceptors) obtained with two subsequent wash/spin cycles with 500-ml aliquots of buffer. Recovery of ^{14}C-labelled vesicles (acceptors) was typically >90%. Control experiments in which donors were labelled with a non-exchangeable marker indicated that all of the donor vesicles bound to the ion exchange column under the conditions of the experiment. Cholesterol accumulation by acceptors was determined using an LS 3801 Beckman scintillation counter equipped with a ^{14}C/^{3}H dual-label program.

Two groups of mice (n=4) were maintained in metabolic cages and faeces collected daily. After 3 days one group was injected with 200 μl of saline and the second group with approx. 6 mg of EPC LUV_{100} (dose 300 mg/kg). Faecal material was collected for a further 7 days. Samples were extracted using an isopropanol/chloroform extraction procedure and subsequently assayed for total cholesterol, free cholesterol and cholesteryl esters, as described above.

Experiments were carried out on mice maintained on regular, laboratory food for rodents (cholesterol excretion rate 10-12 μmol/g faeces) and on Teklad low cholesterol (casein-based diet which resulted in an excretion rate of approx. 0.8 μmol cholesterol/g faeces).

Fig. 1 demonstrates cholesterol mobilization by a homogeneous population of LUV with a mean diameter of 125 nm as determined by QELS (referred to as LUV_{100} and prepared by extrusion as described above). A dramatic increase in plasma cholesterol was observed for animals receiving liposomes (Fig. 1A). Sterol levels peaked 4-8 h after injection at a concentration nearly double that measured in the control mice injected with an equivalent volume of saline. Plasma cholesterol concentrations gradually returned to normal levels after 48 h correlating well with the liposome clearance profile shown in Fig. 1B. Liposomes were labelled with trace amounts of [^{14}C]CHE, a non-exchangeable, non-metabolizable marker frequently used to monitor liposome clearance and distribution in vivo.

Using gel filtration as described above, mouse plasma was fractionated and the cholesterol profile determined using the chemical assay procedure of Rudel and Morris. Plasma from control and liposome-treated animals were compared and the results are shown in Figs. 2A and 2B. Fig. 2A shows a normal cholesterol distribution with the majority of cholesterol associated with combined LDL and HDL peaks (fractions 22-50). The elution volumes of VLDL, LDL and HDL were determined as described above. A minor quantity of sterol was detected in the void volume, corresponding to the larger chylomicron and VLDL lipoprotein particles, but quantitatively these fractions represent <5% of the total cholesterol content of the plasma. The elution profile of plasma from liposome-treated animals (4 h time point) is shown in Fig. 2B. The [^{14}C]CHE liposome marker was almost exclusively detected in the void volume, indicating that the LUV_{100} were well separated from the fractions containing LDL and HDL (liposomes smaller than 100-nm diameter are included in the gel and cannot be separated from LDL). The absence of

radioactivity in the remaining fractions indicated that little, if any, assimilation of vesicles into the lipoprotein pool occurred. However, it is possible that small quantities of vesicles had undergone structural transitions to lipoprotein-like particles, but were removed rapidly from the circulation and therefore, not detected.

The cholesterol content of column fractions, shown in Fig. 2B clearly shows that the excess sterol in the plasma of treated mice is associated with LUV. The slight frame shift of peaks between Fig. 2A and Fig. 2B is the result of differences in elution rate and not due to changes in lipoprotein size. Using TLC analysis it was determined that >90% of the liposomal cholesterol was free cholesterol, the remainder being cholesterol ester.

The excellent separation of LUV_{100} from the quantitatively most abundant lipoproteins enabled straightforward isolation and subsequent analysis of the vesicle lipids. Liposome cholesterol accumulation was shown by the increasing C/P ratio of vesicles over a 24-h time-course in vivo, as shown in Fig. 3. Consequently, after 24 h the liposomes remaining in the circulation (approx. 10-15% of the initial dose) were in equilibrium with respect to cholesterol and net sterol movement was negligible.

Plasma cholesterol concentrations were measured over a 48-h period in animals treated with a variety of liposomal preparations varying in diameter from 30-250 nm. Sonicated vesicles were prepared as described above. The remaining vesicles were produced by extrusion of MLV through filters with defined pore-sizes to give vesicle populations with the mean diameters described above. Vesicles are referred to by the filter pore size used for their synthesis.

The amount of cholesterol accumulated and removed by liposomes in vivo is a function of both the rate of cholesterol uptake and the rate of liposome clearance. An estimate of the mass of cholesterol removed from the circulation (mostly by the RES) can be made by calculating the C/P ratio of vesicles in vivo from plasma concentration of vesicle phospholipid and cholesterol as the excess plasma

concentration above the control at the various experimental time points. All cholesterol above control levels is associated with circulating liposomes. The plasma volume of mice used in these studies was approx. 1 ml, consequently the total amount of phospholipid cleared from the circulation between time points was known. Using the average C/P ratio measured for vesicles between each assay interval an estimate of the amount of cholesterol removed was obtained. The analysis was not continued beyond the point where less than 5% of the initial phospholipid dose remained in the circulation as below this level the measurement error was too large to determine accurate C/P ratios. Fig. 4A shows the cumulative level of cholesterol removed by LUV_{100} up to the time when approx. 5% of the dose remains. After 40 h 2800 nmol of cholesterol were removed from the circulation by the RES, which represents 33 mol% of the injected phospholipid dose. This analysis was used to compare the various liposomal preparations tested. For each preparation the plasma cholesterol and phospholipid clearance profiles were determined and analyzed as described above. The results in Fig. 4B show that LUV mobilize cholesterol most efficiently.

The transfer of sterol from donor vesicles to unilamellar and multilamellar vesicles was studied. Using freeze-fracture electron microscopy and NMR analysis, it has been shown that MLV sized through 400-nm pores retain a number of internal lamellae and therefore cannot be classified as LUV. The transbilayer movement (flip-flop) of cholesterol is rapid, on the order of seconds to minutes in a liquid crystalline bilayer under conditions that promote net sterol flux. Consequently, it was expected that multilamellar systems would act as a good sink for cholesterol as sterol should rapidly disperse through the internal lamellae.

Using an <u>in vitro</u> model in which LUV_{100} or MLV_{400} were incubated with a 10-fold excess of donor liposomes containing tritiated cholesterol as described above, the net transfer of sterol from donor to acceptor was monitored. The rate of cholesterol accumulation in the unilamellar preparation was greater than that observed for the

oligolamellar vesicles. It is interesting to note that in the presence of a 10-fold excess of donor vesicles the equilibrium C/P ratio of the acceptor should be approx. 0.9:1. The data in Fig. 5 show that the 100-nm acceptors only achieve a ratio of 0.35:1 after 8 h at 37°C. This is approximately half the rate of accumulation observed for the same vesicles in vivo (Fig. 3).

The cholesterol mobilizing properties of two types of LUV_{100} were compared. The two types of LUV_{100} were composed of EPC/EPG (95:5 mol ratio) which is liquid-crystalline at 37°C and DSPC/DSPG (95:5), a gel-state lipid matrix at the body temperature of the mouse. Phosphatidylglycerol (PG) was incorporated to impart a surface negative charge, necessary to prevent the gel-state vesicles from aggregating in the absence of cholesterol as described in Nayer et al., Biochim. Biophys. Acta, 986:200-206 (1989), incorporated herein by reference. Reliable comparison of the two systems was facilitated by adding a negative charge to the EPC vesicles. The results, presented in Fig. 6A, reveal that the gel-state vesicles produced a delayed increase in plasma cholesterol which did not peak until after 24 h, whereas EPC/EPG vesicles gave rise to a cholesterol profile similar to that observed for EPC alone.

The data in Fig. 6A demonstrate that the rate of cholesterol accumulation for these two types of vesicle was the same. The different plasma cholesterol profiles occurred because approximately 70% of the DSPC/DSPG vesicles were cleared within 4 h compared to less than 30% of the EPC/EPG LUV_{100} (Fig. 6B). The bulk of cholesterol mobilization occurred in the first 24 h, consequently liquid crystalline EPC/EPG removed more than 3000 nmol to the RES, whereas DSPC/DSPG vesicles removed 1700 nmol. The source of the accumulated liposomal cholesterol and its fate was determined. Ultimately, cholesterol efflux must occur from atherosclerotic plaque to achieve regression. However, it is known that the cholesterol within cells and atherosclerotic lesions equilibrates more slowly than sterol present in plasma membranes directly exposed to acceptor particles. Movement of

this cholesterol will be a secondary event initiated by the primary efflux of outermembrane cholesterol.

In a 20-g mouse approximately 35% of the circulating sterol is associated with lipoproteins and about 65% with the plasma membranes of erythrocytes. However, all of the sterol associated with erythrocytes is free cholesterol, whereas a large proportion of lipoprotein sterol is esterified. Consequently, the largest pool of free cholesterol in the circulation is in the red blood cell plasma membrane. It was found that this source of cholesterol does not change significantly in the presence of liposomes, despite a two fold increase in plasma sterol concentration. This result is shown in Fig. 7A.

Erythrocyte membrane cholesterol can be depleted by liposomes in vitro. Consequently it was determined whether erythrocytes act as the primary sterol donor and then rapidly replenished by lipoproteins which are in turn able to extravasate and scavenge more sterol from peripheral tissues. Erythrocytes were isolated from mice and labelled with [^{3}H]cholesterol in vitro. The labelled cells were injected into a group of mice, half of which were subsequently treated with saline and half with 300 mg/kg of EPC LUV_{100}. The specific activity of red blood cell cholesterol was determined over an 8-h time-course and the two groups compared. As demonstrated in Fig. 7B, the decrease in cholesterol specific activity is the same for both the control and experimental group. Interpretation of these data is limited by the fact that cells labelled in vitro are also removed from the circulation over a similar time-course (determined by chromium labelling). However, it can be estimated that at least 50% efflux of cell sterol would be necessary to account for the rise in plasma cholesterol observed after 8 h. This would result in a considerable dilution of erythrocyte cholesterol if this sterol pool were continuously replenished. As this has not been observed, the data suggest that red blood cell cholesterol is not the primary source of the liposomal sterol accumulated in vivo.

C/P ratios of lipoproteins showed a significant decrease over control values in the first 8 h (Fig. 7C). The ratio returned to normal values after 8 h mirroring the time-course of cholesterol accumulation by vesicles. This suggests that it is primarily lipoprotein cholesterol in equilibrium with circulating liposomes, and that lipoproteins mediate the transfer of cholesterol from peripheral tissues to liposomes. The results are also consistent with observations in vitro that indicate cholesterol can undergo desorption from lipoproteins more readily than from erythrocytes. Finally, the rate of cholesterol accumulation by LUV_{100} in vivo (Fig. 3) is considerably faster than that observed in vitro (Fig. 5), indicating that the rate of cholesterol desorption from sources in vivo is greater than from the 100 nm vesicle donors used to obtain the data in Fig. 5.

Example 2 -- Regression of Atheromas in Rabbits Treated with Liposomes

This example demonstrates mobilization of cholesterol and regression of atheromas in rabbits treated with liposome compositions of the present invention. Plasma cholesterol concentration increased 2.5 times in liposome treated rabbits. Aortic lipid content decreased 25% in liposome treated animals.

Egg phosphatidylcholine (EPC) was supplied by Princeton Lipids (Princeton, NJ). A 0.5% cholesterol supplemented diet was obtained from Teklad Premier. Blood collection tubes and butterfly needles (23 gauge) were from Becton-Dickinson (Missisauga, Ontario). Ketamine, xylazine, heparin, Innovar and Euthanyl were supplied by MTC Pharmaceuticals, Janssen Pharmaceutics and Organon Technika (Ontario). Bio-Gel A-15m was purchased from Bio-Rad. Prepacked Solid Phase silica gel columns were acquired from Burdick & Jackson. All chemical and solvents were of analytical grade from BDH Chemicals (Vancouver, B.C.)

Forty eight New Zealand White (NZW) rabbits were housed in wire cages at the Animal Unit of the Research Centre conforming to guidelines set by the Canadian Council on Animal

Care and the University of British Columbia. The animals were maintained in a controlled temperature environment with a 12 hour dark/light cycle. Approximately 150g of food were given per animal per diem. Water was freely given.

Lesions induced in rabbits as a result of maintaining the animals on cholesterol enriched diets for more than two months, do not regress for lengths of up to two years even when they are returned to standard rabbit chow. St. Clair, Prog. Cardiovasc. Dis., 26:109-132 (1983). Even after cessation of cholesterol enriched diets, lesions have been noted to progress and increase in complexity. Prior et al., Arch. Path., 71:82-94 (1961). Moreover, in cases where intermittent feeding schedules were administered or a low cholesterol-enriched diet was given over a period of years, lesions similar to the calcified ulcerated lesions observed in humans have been produced. Constantinides et al., Arch. Pathol., 70:81-92 (1961).

The correlation between hypercholesterolemia and the onset and progression of atherosclerosis in the rabbit is well established. St. Clair, supra. To ensure that an equal distribution of animals were divided into the respective treatment groups, careful pairing of the animals was done. Initially, the 48 NZW weanlings were screened for responders to the 0.5% cholesterol enriched diet (Teklad diet 0533). The animals were fed the cholesterol diet for one week and plasma cholesterol concentrations monitored until returning to normal. Animals were matched by the extent of the rise in plasma cholesterol levels as well as the rate at which the levels returned to normal. This enabled an equal distribution of animals to be placed into two groups of 24 that were fed either standard rabbit chow or 0.5% cholesterol enriched rabbit chow for 20 weeks to induce atherosclerotic plaque formation. During this time, plasma lipid levels were monitored on a monthly basis. Two animals were euthanized due to complications probably associated with handling and were excluded from the final analyses. After the diet induction period, five animals from each group were sacrificed to verify the formation of lesions and serve as the standards against

which the effectiveness of liposomal treatment was assessed. Thereafter, all remaining animals were fed regular rabbit chow until the conclusion of the study.

Rabbits were fed a 0.5% cholesterol-enriched diet for 20 weeks in order to induce intermediate lesions more significant than fatty streaks associated with shorter duration cholesterol-enriched diets. Chemical and histological analyses of aortas obtained from rabbits following the diet induction period, but prior to treatment, revealed plaques formed that were rich in lipid and surrounded by fibrous tissue. These plaques consisted of almost equivalent amounts of cholesterol and cholesterol ester. The aortic phospholipid in these animals was 15±4 μmol/g wet tissue and aortic total cholesterol was 114±28 μmol/g wet tissue (61±13 μmol/g cholesterol and 53±15 μmol/g cholesterol ester). Animals maintained on a standard diet had aortic phospholipid levels of 4±0.3 μmol/g wet tissue and aortic total cholesterol levels of 10±1 μmol/g which was predominantly cholesterol. The degree of surface plaque involvement in cholesterol fed animals was 78±14%.

Based on the pairing of plasma cholesterol concentrations, 18 rabbits remaining from each diet group were separated into groups of 9 and were treated with EPC LUV_{100} at a dose of 300 mg/kg or the equivalent volume of saline. Treatment was initiated 4 weeks after return to standard rabbit chow and was given over a 100 day period. The treatment consisted of ten bolus injections of phospholipid or saline administered into the marginal ear vein. One injection was given every 10 days.

The rabbits ranged from 4-6 kg in weight. Each treatment of the vesicle-receiving rabbits required the preparation of approximately 150 mls of LUV_{100} at a concentration of 200 mg/ml. Typically, 6 gram aliquots of EPC were hydrated with 30 ml of filtered 150 mM NaCl, 20 mM HEPES (HBS), pH 7.4, in sterile 50 ml conical tubes, vortexed and kept overnight . As described in Example 1 above,, the resulting multilamellar vesicles (MLVs) were used to generate LUV_{100} by extrusion through two stacked polycarbonate filters

of 100 nm pore size using a 10 ml water-jacketed thermobarrel Extruder (Lipex Biomembranes, Vancouver, B.C.), according to the method of Hope et al., Biochim Biophys. Acta, 812:55-65 (1985), incorporated herein by reference. Vesicle sizes were determined by quasi-electric light scattering (QELS) analyses utilizing a Nicomp Model 370 submicron laser particle sizer (Pacific Scientific, MD). The vesicles used for the 10 treatments had an average diameter of 114±7 nm.

A small dose of Innovar™ was given to promote calmness and vessel dilation in animals to ease routine bleedings necessary for plasma lipid analyses. To facilitate the final blood collections, ketamine (40 mg/kg) and xylazine (8 mg/kg) were given intramuscularly to sedate the animals. Fifty units of heparin (Hepalean) followed by a lethal dose of phenobarbital (Euthanyl) were then perfused into the marginal ear vein before laparotomy. Organs were removed, rinsed in saline and immediately frozen in liquid nitrogen. The heart and full length aorta were collected in one section and kept in iced saline. The animals were sacrificed in groups of 8-10 on alternate days. The organs were randomized prior to processing and analyses.

Each aorta was separated from the heart at the aortic valve and was carefully cleaned to remove any adherent adventitial fat. The aortas were cut along the ventral surface, opened, and photographed on a black background. The photographs were used in conjunction with the negatives to aid in the collection of digitization data as well as to facilitate the division of the aortas into three regions: the arch, thoracic, and abdominal aortic segments as described by Rosenfeld et al., Atherosclerosis, 8:338-347 (1988), incorporated herein by reference. Nine animals were in each of the 4 treatment groups: (1) vesicle-treated cholesterol-fed animals (VC), (2) saline-treated cholesterol-fed animals (SC), (3) vesicle-treated normal diet animals (VN) and (4) saline-treated normal diet (SN). Six aortas from each group were allocated for lipid analyses and stored at -20°C until analysis. The remaining three samples in each group were fixed in 10% neutral buffered formalin for at least 48 hours

and used for gross staining with Sudan IV and histology. Holman et al., Lab. Invest., 7:42-47 (1958), incorporated herein by reference. At the time of lipid analysis, the aortas were patted dry and divided into the three segments. Wet weight and length were measured and the aortic segments were homogenized (Polytron) in HBS. Two additional washes of the Polytron probe with HBS were collected for each segment to ensure complete homogenate recovery.

Whole aortic segments were analyzed by digitization. In this analysis, photographic negatives obtained from all unstained aortas were illuminated generating an image using a Microcomputer Imaging Device (Imaging Systems). The percentage of plaque involvement was calculated by dividing the area occupied by surface plaque by the area of the entire aorta segment. Distinct differences were observed in the degree of shading of plaques and uninvolved aortic tissue. Assessments of the percentage of atherosclerotic plaque involvement were performed by two observers and the results were averaged. Interobserver variation was within ±5%.

Cholesterol and phospholipid content of the aortas and livers of the sacrificed animals were quantified following Bligh and Dyer extractions of the homogenates. Bligh and Dyer, Can. J. Biochem. Physiol., 37:911-917 (1959), incorporated herein by reference. Total cholesterol, cholesterol, and cholesterol ester contents were determined according to the method of Rudel and Morris, J. Lipid Res., 14:364-366 (1973), incorporated herein by reference. Cholesterol and cholesterol esters were separated by silica gel chromatography on Burdick and Jackson prepacked 200 mg Solid Phase Silica Gel columns. Cholesterol esters were eluted with 1 ml methylene chloride. Cholesterol was collected following methylene chloride/methanol (95:5) elution after transferring the columns to a new carrier. Phospholipid content was measured according to Fiske and Subbarow, J. Biol. Chem., 66:375-400 (1924), incorporated herein by reference. Lipoprotein lipid profiles were quantified by enzymatic procedures after phosphotungstic acid precipitation.

Aliquots of aorta or liver homogenates were incubated overnight at 37°C with 1 ml of 1N NaOH. Thereafter, sodium dodecylsulphate (SDS) was added to the mixture to make a 1% solution needed to solubilize any remaining particulate matter. Protein content of the samples was quantified by the bicinchoninic acid (BCA) protein assay method (Pierce Chemical Company, Rockford, IL) after incubation for 1 hour at 60°C and read at A_{562} against an albumin standard.

Typically, 2-3 mm segments from the arch, thoracic, and abdominal aorta of three different animals within each treatment group were divided into left and right halves and embedded in paraffin. At least 8 segments from each region were prepared as blocks, depending on the length of the aorta. Alternate sections of 5 μm were adhered to gelatin coated slides from paraffin blocks and visualized with hematoxylin and eosin (H&E) or Weigart's-van Gieson's stains. Intima/media ratios of the different regions were calculated by initially measuring an average ratio from 3 photographs generated from each section and using this value to determine a final mean±standard deviation from all the sections made from the animals of each group.

The nature of plaques from animals sacrificed after the diet induction period, but prior to any treatment was examined after sections were made from segments held into place with tissue mount (OCT) on wooden stages and quick frozen in isopentane followed by liquid nitrogen. Subsequently, alternate sections of 5 μm were adhered to polylysine coated slides and visualized with Sudan IV differentiated with Harris' hematoxylin, H&E or van Gieson's stains to highlight lipids and collagen.

Unless otherwise indicated, mean±standard deviation values are presented. The significance of the difference of the means was assessed by an analysis of variance using the two-sample t test. Only values of $P<0.05$ were considered significant.

During the course of this study, animals maintained on the atherosclerotic diet exhibited plasma total cholesterol concentrations ranging from 5-10 times that of the control

animals fed the standard diet while fed the cholesterol-enriched diet. The cholesterol concentrations remained elevated (2-5 times higher) until the conclusion of the study even though standard rabbit chow was given during the treatment period. This is illustrated in a typical time course of cholesterol mobilization resulting from the infusion of 300 mg/kg EPC LUV_{100} or an equivalent volume of saline demonstrated in Fig. 8. A comparison of control animals injected with saline demonstrates that animals previously fed the high cholesterol diet (panel A) maintained plasma cholesterol concentrations 3 times higher than animals maintained on the standard diet throughout the study (panel B) even though the cholesterol diet was terminated 10 weeks earlier. Despite the atherosclerotic animals having excess plasma cholesterol, an injection of LUV_{100} resulted in a dramatic 2.5 times increase in plasma cholesterol concentrations in both hyper- and normocholesterolemic animals when compared to saline treated counterparts. Plasma cholesterol levels peaked at 24 hours post-infusion before returning to baseline levels after 5 days. This time course correlates with the removal of vesicles from the circulation measured as total plasma phospholipid concentration illustrated in the clearance profiles shown in Fig. 9. Although atherosclerotic animals had slightly higher total phospholipid concentrations, similar clearance kinetics of the injected vesicles were seen between normal and hypercholesterolemic rabbits.

As demonstrated in Example 1 above, the amount of cholesterol accumulated and removed by liposomes with each infusion is a function of the rate of liposomal cholesterol uptake and the rate of vesicle clearance. Also, it was determined that all cholesterol above saline treated levels was associated with circulating liposomes by generating a cholesterol and phospholipid profile after separating vesicles from plasma by gel filtration. This showed that excess plasma cholesterol was associated with the vesicles and that >90% of the cholesterol was free cholesterol. Hence, an estimate of the mass of cholesterol removed from the circulation (mostly

by the RES) was made by calculating the C:P ratios of vesicles at intervals following each injection from plasma phospholipid concentrations (vesicle-treated concentration minus saline-treated concentrations) and cholesterol (excess plasma concentration above the control concentration) at different time points during the experiments.

The plasma volume of the rabbits was approximately 150 ml. An estimate of the cholesterol removed was calculated employing the average C:P ratio measured for vesicles at each assay interval. This data is shown in Fig. 10. The data represents an average±standard deviation expressed as mmol of cholesterol removed with each treatment in hypercholesterolemic animals and was calculated from data obtained from treatments 1, 4 and 10. The analysis was not continued beyond the point where less than 10% of the initial phospholipid dose remained in the circulation. Below this level, the measurement error was too large to determine accurate C:P ratios. After 104 hours it was estimated that approximately 1 mmol of cholesterol was removed from the circulation by the RES, which represents approximately 50 mole % of the injected phospholipid dose. Furthermore, based on plasma cholesterol concentrations measured in animals 24 h post-injection, each of the 10 infusions of liposomes caused dramatic cholesterol mobilization.

The ability of the animals to tolerate and remove repeated injections of phospholipid and the consequences of administering excess phospholipid on plasma lipid levels were examined. Chronic short term (one week) administration of Intralipid, an emulsion of triglycerides and phospholipids, causes increased LDL levels. Although the phospholipid content of Intralipid is comparable to the dose of 300 mg/kg LUV_{100} per injection of the present treatment regimen, Intralipid is generally given intravenously on a daily basis as a nutritional supplement.

Each injection of 300 mg/kg EPC LUV_{100} apparently induces a transient 100-fold increase in plasma phospholipid concentrations and at the end of liposomal therapy (10 injections) each animal received an average total dose of

12-20 mmol (10-15g) of phospholipid. The clearance profiles of several injections of EPC LUV_{100} in cholesterol fed rabbits is shown in Fig. 11A. As illustrated, significant differences in the rates of vesicle clearance between injections were not detected. Fig. 11B shows that similar concentrations of vesicle phospholipid remain in the circulation 24 h post-injection in both normo- and hypercholesterolemic animals following serial injections. If the ability of the fixed macrophages of the RES were compromised, increasing phospholipid levels would likely be detected during the later treatments. Furthermore, 5 days post-injection, the injected dose of liposome phospholipid was completely removed from the circulation and plasma phospholipid and cholesterol concentrations returned to baseline levels.

At the conclusion of the study, saline-treated cholesterol-fed animals maintained elevated plasma cholesterol levels whereas vesicle-treated animals had levels comparable to animals maintained on the standard diet. The reduction in plasma cholesterol concentrations of vesicle-treated atherosclerotic animals resulted from a reduction in both plasma LDL and HDL cholesterol concentrations although the relative proportions of HDL/LDL cholesterol were not affected. No changes in the plasma lipid profiles (cholesterol, phospholipid or triglycerides) were detected in animals maintained on standard rabbit chow throughout the study. Plasma phospholipid levels in vesicle-treated animals were similar to their saline-treated counterparts despite the injection of approximately 15 grams of phosphatidylcholine per animal during liposomal therapy. These results, unlike those observed with Intralipid infusions, suggest that repeated administration of LUV_{100} given at 10 day intervals does not compromise RES function or normal plasma lipid homeostasis.

Erythrocyte cholesterol remained constant throughout the infusions. However, a decrease in the C:P ratios of lipoproteins was detected over the first 24 hours. This C:P reduction gradually returned to normal levels after 48 hours (see Fig. 12). This time course mirrors cholesterol accumulation by the vesicles. These results suggest that the

lipoprotein pool of cholesterol rapidly equilibrates with the vesicles and supports the hypothesis that liposomes generate cholesterol-poor lipoprotein particles that can access peripheral tissues and promote cellular cholesterol efflux.

The extent of lesion progression or regression was assessed by three complementary methods: (1) chemical lipid and protein assays to determine lesion bulk, (2) digitization of gross surface morphology to quantitate the degree of plaque involvement, and (3) histochemistry to examine the nature and depth of the lesions.

Despite elevated plasma cholesterol concentrations persisting in animals returned to standard rabbit chow, saline-treated animals were found to have arterial wall cholesterol content expressed per gram wet weight of 94±12 μmol/g total cholesterol, 58±6 μmol/g free cholesterol and 37±9 μmol/g cholesterol esters with an average surface plaque involvement of 77±17%. Although there appears to be slight reduction in the cholesterol ester content, the values of the lipid content of saline-treated animals were not significantly different from values found in atherosclerotic animals prior to treatment indicating that there was no progression or regression of lesions after 4 months. On the other hand liposome-treated animals were found to have significantly less cholesterol content of the entire aorta with levels of 85±8 μmol/g total cholesterol, 48±5 μmol/g free cholesterol and 37±6 μmol/g cholesterol esters. Because there were no significant differences between the lipid content of animals before or after saline treatment, the reductions in plaque cholesterol content between liposome- and saline-treated animals indicates regression, not simply decreased progression, of plaques.

Aortic lipid content was expressed per gram of protein weight as wet weights are likely to be more variable. No significant differences were found between the protein levels in both saline- and vesicle-treated animals. The protein content of the aortas to be 0.41 g protein/g wet weight and 0.43 g protein/g wet weight, respectively. Expressing the data per g protein, liposomal therapy resulted

in a 25% reduction in total cholesterol content of the entire aorta of vesicle-treated animals compared to saline-treated controls. By segment, there was a 48% reduction seen in thoracic aorta cholesterol levels and small reductions in the arch and abdominal aortas (see Fig. 13A). Significant reductions in the cholesterol ester levels in vesicle-treated animals were also noted and again the thoracic aorta demonstrated the greatest decrease (see Fig. 13B). In addition to decreased cholesterol content, aortic phospholipid levels in vesicle-treated atherosclerotic animals decreased, although not to the level of statistical significance.

In order to maximize the number of animals within each group, all negatives generated from photographed unstained aortas were digitized. Gross Sudan IV staining of 3 aortas from each treatment group confirmed the same degree of surface plaque involvement as unstained aortas. The area of plaque involvement was determined by digitization. The data is shown in Fig. 13C. Liposome-treated, cholesterol fed rabbits demonstrated 61±13% involvement of the entire aorta compared to 77±17% involvement of saline-treated animals, representing an overall 16% reduction of surface plaque. In agreement with the reductions in cholesterol content detected by lipid analyses, the thoracic aorta exhibited the most benefit from liposome infusion with digitization analysis and displayed a 26% reduction in plaque involvement, whereas the abdominal aorta revealed a 16% reduction. There was a slight reduction in the degree of surface plaque involvement of the arch that failed to reach statistical significance. No significant differences between treated and untreated control animals maintained on the standard diet were seen and both groups showed essentially no plaque involvement.

Histochemical analysis revealed extensive raised plaques (intimal thickening) in the cholesterol fed animals as expected from gross surface morphology inspection. Whereas digitization quantitated the extent of plaque involvement, histochemical analyses allows the depth and nature of the lesions to be assessed. Generally, the plaques exhibited extensive intimal thickening due to stratified lipid deposits

that were surrounded by a collagenous network. The arch region was noted to display more advanced lesions of apparent crystalline cholesterol deposits and showed a few isolated necrotic foci as detected with H&E staining.

Representative sections of the thoracic aorta of vesicle-treated and saline treated animals revealed that lesions of animals treated with vesicles manifested fewer lipid deposits and showed moderately reduced plaque thickening when compared to saline treated atherosclerotic animals. This is quantified in Table 1 summarizing the data obtained from the analysis of pictures taken from multiple sections used to assess the severity of lesions present in the arch, thoracic or abdominal aorta of atherosclerotic animals. As can be seen, a decrease in the intima/medial ratios in the arch and thoracic regions of liposome treated animals were detected, whereas no changes were detected in the abdominal aorta. No apparent differences were detected between treated and untreated animals maintained on the standard diet throughout the study.

Cholesterol feeding of rabbits often leads to the accumulation of cholesterol in a number of tissues including the liver. However upon the return to regular rabbit chow, non-arterial tissue cholesterol levels often revert to normal within a month. Liver cholesterol content was measured in order to gain insight into whether (1) increased biliary excretion of cholesterol might be occurring in liposome-treated animals due to massive deposition of the injected phospholipids in the liver resulting in reduced liver cholesterol levels or (2) there was a detrimental accumulation of cholesterol mobilized by the liposomes to the liver. In atherosclerotic animals, liposome-treated rabbits demonstrated a slight reduction in liver cholesterol content having average levels of 8 μmol/g that are comparable to control animals fed the standard diet. Saline-treated animals exhibited average levels of 11 μmol/g. This difference was not statistically significant.

All publications, patents and patent applications mentioned in this specification are herein incorporated by

reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Table 1. Measurement of Intimal/Medial ratios in the different regions of the aorta of vesicle and saline treated atherosclerotic animals.

Intimal/Medial Ratios

Portion of aorta	Liposome Treated	Saline Treated	Significance (P value)
Arch	1.51±0.55	1.76±0.94	N.S.
Thoracic	1.34±0.73	1.93±1.12	P< 0.01
Abdominal	1.84±0.95	1.81±1.25	N.S.